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12. Applied Aspects of Ectomycorrhizae

Introduction

Mycorrhizae, or fungus-root associations, are the norm for most vascular plants (15). Many plants depend on their mycorrhizal structures for adequate uptake of nutrients and survival in natural ecosystems (11, 48, 81). Various types of mycorrhizae are known, but this paper will address only ectomycorrhizae and their applied aspects. Ectomycorrhizal hosts include species primarily in the Pinaceae, Fagaceae, Betulaceae, and Saliceae and a few genera in other families, e.g., *Eucalyptus*, *Tilia*, and *Arbutus* (47); several members of the Caesalpiniaceae and Dipterocarpaceae are prevalent ectomycorrhizal hosts in the tropics (61). Numerous fungi, mostly higher Basidiomycetes and Ascomycetes, form ectomycorrhizae (70, 79); a few Zygomycetes in the Endogonaceae are also involved (12).

Three major features characterize the ectomycorrhiza structure: 1) fungus colonization or sheathing of the short feeder roots, the fungus mantle; 2) intercellular fungus penetration between cortical cells, the Hartig net; and 3) morphological differentiation of colonized feeder roots via increased branching and elongation (Plate 1, A–F). This final characteristic together with the often dense mycelial connections with the soil allow for a highly active absorptive organ which colonizes large volumes of soil. Thus, a major benefit to the host is increased water and nutrient uptake, particularly of immobile ions such as phosphates. Other benefits include increased tolerance to drought, high soil temperatures, soil toxins, and extreme pH, as well as protection against root pathogens (25, 28, 36, 81). In return, the fungi depend on their hosts for carbohydrates, mostly in the form of simple sugars and vitamins. The physiology and ecology of ectomycorrhizae have been extensively reviewed (22) and will not be attempted here.

Much of our understanding on the functions of ectomycorrhizae has come from research directed towards practical application in forestry. Early in this century, for example, repeated failures in establishing exotic pine planta-

tions in the tropics and other areas where ectomycorrhizal hosts do not naturally occur clearly demonstrated the dependence of these trees on their fungal symbionts. Only after inoculation with forest soil containing ectomycorrhizal fungus propagules could these trees survive and function properly (48, 49). Intensive mycorrhiza research in the past 30 years has increased our understanding of the complex physiology and ecology of ectomycorrhizae and our appreciation of the role of symbiosis (22). Most importantly, this information provides many necessary tools and concepts for strengthening forestry programs around the world.

Today, widescale inoculation of forest nurseries with selected ectomycorrhizal fungi appears imminent. Commercial interest in producing pure cultures of ectomycorrhizal fungus inoculum expands the possibilities of worldwide application. The success of these inoculation programs hinges on selection of effective and beneficial fungal symbionts. Little data exist on which of the thousands of possible ectomycorrhizal fungi may be the best candidates for inoculating a particular host species. Other considerations, such as nursery management practices and location of outplanting sites, complicate the selection process. New inoculation programs must be strongly research oriented from the outset (80).

In this paper we briefly review the considerations involved in determining the need for ectomycorrhizal inoculation, the techniques available for inoculation, and some relevant criteria for selecting specific fungi for inoculation. The readers are referred to the comprehensive reviews of Mikola (48, 49) for historical perspectives of worldwide development and applications, Trappe (80) for selection of fungi for nursery inoculation, and Marx (31) for detailed information on current inoculation techniques.

Forestry uses of ectomycorrhiza inoculation

Ectomycorrhizal host trees must be accompanied by their mycorrhizal fungi to survive when planted in areas lacking suitable fungi. This has been experienced many times in the introduction of exotic pines into the Southern Hemisphere and tropical islands (6, 48). Afforestation attempts in the treeless grasslands of the U.S.A. and the steppes of Russia have also required inoculation for success (18, 45). Schramm (66) and Marx (26, 30) have shown the absolute requirement for ectomycorrhizal planting stock for tree establishment on stripmined lands and other severely perturbed sites.

Although successful inoculation of tree seedlings already planted in the field have been reported (6), nursery inoculation is more common. Seedlings inoculated in the nursery can establish a healthy ectomycorrhiza system before outplanting. The increasing use of soil fumigation to eliminate pests in nurseries makes mycorrhizal inoculation of nurseries especially critical. Complete soil fumigants, such as the commonly used methyl bromide-chloropic-

rin* mixes, can thoroughly eradicate ectomycorrhizal fungus populations (4). Tree seedlings lacking ectomycorrhizae suffer severe nutrient deficiencies early in their first growing season; the deficiencies persist until mycorrhizae are formed (43, 82). Although deleterious to resident fungal populations, nursery fumigation is necessary for present inoculation techniques; competition from resident ectomycorrhizal fungi is eliminated or reduced as a prerequisite for successful establishment of the selected fungal inoculum.

New nurseries often show mycorrhizal deficiency symptoms, particularly when established on heavily fumigated former agricultural land. Trappe and Strand (82) report a striking example of this in the Willamette Valley of Oregon. The first crop of Douglas-fir seedlings exhibited severe phosphorus deficiency, unexpected because soil analyses had indicated no such deficiency. Subsequent heavy fertilization with phosphates did not alleviate the deficiency. Only after natural inoculation by windblown spores did the seedlings recover and begin to grow (Plate 2, A). A similar example was described by Marx *et al.* (43) for an Oklahoma nursery established on former pasture with few nearby ectomycorrhizal host trees. Significant improvement in seedling quality occurred after inoculation with pure fungus cultures. Substantial economic losses and disruption of forestation programs can result from failure to recognize and correct mycorrhiza deficiencies in nurseries.

The millions of containerized seedlings produced around the world offer another situation in which ectomycorrhiza inoculation may be important. Many cultural practices used in raising containerized seedlings—e.g., artificial potting mixes, frequent applications of concentrated soluble fertilizers, and greenhouse rearing—minimize or severely retard ectomycorrhiza formation. Most containerized seedlings we have examined appear vigorous and healthy but routinely lack mycorrhizae. We suspect that the mycorrhizal root system will increase survival and initial growth of containerized seedlings after outplanting. Ruehle (62) recently reported better survival and growth of containerized loblolly pine seedlings inoculated with *Pisolithus tinctorius* than of nonmycorrhizal seedlings planted on severely disturbed soil. Interactions between inoculation and the cultural practices mentioned above are currently under intensive investigation.

Ectomycorrhiza inoculation of tree seedlings offers promise for increasing reforestation success of suboptimal sites. Inoculation of seedlings destined for cutover lands has sometimes been thought unnecessary due to the abundance of mycorrhizal fungus propagules still present. We have found this to be true for high quality sites in the Oregon Coast Ranges (Molina and Trappe, unpublished data). Reforestation of sites stressed by drought, heat,

*This paper reports work involving pesticides. It does not include recommendations for operational use nor does it imply that uses discussed here are registered unless specifically stated. All uses of pesticides in the U.S.A. must be registered by appropriate State and Federal Agencies before they can be recommended.

or cold, however, will be improved by planting seedlings inoculated with fungi specifically adapted to those habitats. In droughty sites, for example, the density of fungal propagules may be low due to the frequent failure of a fungal fruiting season. Marx (31) also emphasizes that temporarily adverse conditions are common to many forest sites following timber harvest and site preparation. Harvey *et al.* (16, 17), recently found that both partial and total cutting of timber, followed by broadcast burning, reduced the ectomycorrhizal activity. The resulting delay in ectomycorrhiza formation on non-mycorrhizal seedlings or seedlings mycorrhizal with fungi not adapted to such sites may well decrease seedling survival or growth. Marx *et al.* (39) recently reported that inoculation with the fungal symbiont *Pisolithus tinctorius*, significantly increased growth and survival of five southern pine species planted in various sites in North Carolina and Florida. Much research is still needed on selection of ectomycorrhizal fungi for specific reforestation sites.

Techniques of inoculation

Once the need for inoculation is recognized, several methods are available. Spontaneous inoculation from windblown spores may suffice for nurseries established near stands of ectomycorrhizal host trees. Usually, however, spontaneous inoculation is erratic. When sowing follows spring fumigation, the chances for adequate natural inoculation during the first growing season is slight in regions of summer drought. In the Pacific Northwest of the U.S.A. and many other regions, for example, the height of the fungal fruiting season comes with the onset of fall rains and tapers off over winter and into spring; inoculum density of wind disseminated spores is relatively low in the spring.

Relatively few species of ectomycorrhizal fungi commonly inhabit nurseries. *Thelephora terrestris*, *Laccaria laccata*, and *Inocybe lacera* are common in Douglas-fir nurseries in the Pacific Northwest; *Thelephora* spp. frequently dominate in conifer nurseries around the world. These fungi are aggressive and well adapted to the cultivated, highly fertile, irrigated nursery conditions. Such nursery fungi may not be well suited to many planting sites (31, 80). Other common nursery fungi in temperate regions are the Discomycetes that form ectendomycorrhizae (19). These mycorrhizae are infrequent in natural forests, so their effectiveness is questionable for forest sites.

Four primary sources of inoculum for use in nurseries are listed below with their primary disadvantages (31, 47, 80). The advantages of each are then elaborated, but the promising technique of inoculation with pure cultures is particularly emphasized.

I. Soil inoculum
   1. Large amounts are needed (10 per cent volume).
   2. Pests and pathogens may be introduced, although in past experience this has not proved to be a problem.

3. Fungal symbionts are unknown.

II. Mycorrhizal nurse seedlings interplanted in seedbeds
1. Pests and pathogens may be introduced.
2. Mycorrhizal colonization is uneven.
3. Fungal symbionts are unknown.

III. Spores and sporocaps
1. Collection is seasonally limited and yearly availability unpredictable.
2. Adequate quantities of sporocarps may be difficult to obtain.
3. Much hand labor is needed in sporocarp collection.
4. Spore viability is difficult to determine; long-term storage requirements are unknown.
5. Several weeks are needed for mycorrhiza formation after inoculation.

IV. Pure cultures
1. Isolation of some ectomycorrhizal fungi is difficult.
2. Most of the fungi grow slowly in pure culture.
3. Production of sufficient inoculum is time consuming and expensive.
4. Conditions for survival of fungal inoculum in the soil are poorly known.
5. Effective and beneficial fungal symbionts must be selected.

The most commonly used and probably the most reliable ectomycorrhizal inoculum is soil taken from beneath ectomycorrhizal hosts (49). About 10 percent by volume of soil inoculum is incorporated into the top $\pm$ 10 cm of nursery beds prior to sowing or transplanting. Soil inoculum can also be added to the planting hole when seedlings are outplanted. Soil inoculation has been instrumental in establishment of exotic pine plantations in the Southern Hemisphere and continues as a regular practice there today (48).

A major disadvantage of soil inoculation is the relatively large amount of soil needed and its transportation over long distances. Inoculation of new or fumigated beds with soil from established beds is often feasible within individual nurseries. A possible danger with soil inoculum is the introduction of pathogens and other pests, although this has not generally been a problem (80). Nursery managers are nonetheless often reluctant to incorporate nonfumigated soil into fumigated beds. Soil inoculation remains a reliable method of eliminating mycorrhizal deficiencies and deserves further research attention.

Planting mycorrhizal "nurse" seedlings or incorporating chopped roots of ectomycorrhizal hosts into nursery beds as a source of the fungi for neighboring young seedlings has been successful (48, 49) (Plate 2, B and C). Mycorrhizal colonization, however, may spread slowly and unevenly. Chevalier and Grente (7) were able to inoculate seedlings with the highly prized truffle fungus *Tuber melanosporum* by use of nurse seedlings already mycorrhizal with this fungus. The major disadvantages of this method parallel those of soil inocula-

tion—the possibility of introducing unwanted pathogens and other pests, and, the identities and effectiveness of the introduced fungi are unknown. In modern, mechanized nurseries, the presence of large nurse seedlings may interfere with cultural practices.

Basidio- and ascospores or crushed sporocarps have been used occasionally as inoculum, usually in small experiments. Some investigators have reported good success with this technique (10, 27, 34, 42, 63, 65, 73, 75). Asexual spores and sclerotia are further sources of inoculum (21, 76). Theoretically, the use of spores would most closely imitate natural inoculation. Practical application is limited, however, by the generally short season for collecting sporocarps in quantity. In some regions, adverse weather may even eliminate the collecting season. Also, in many regions the fungi fruit in fall whereas the nurseries fumigate and sow seeds in the spring. Spore inoculum then must be stored over winter, but little is known of the storage requirements of ectomycorrhizal fungal spores. This is complicated further by the difficulty of germinating sexual spores of most ectomycorrhizal fungi.

The Gasteromycetes (puffballs and related fungi), with abundant spore masses, offer better sources of large numbers of spores than the gilled fungi. Most recent research has been with *Pisolithus tinctorius* (27, 34, 42, 63, 65). Billions of basidiospores can be easily collected from this large puffball, which often fruits in abundance. Inoculum rates of $5.5 \times 10^8 - 1.3 \times 10^{10}$ spores/m^2 of soil surface have been used to successfully inoculate several bareroot nurseries in southern United States (31). The powdery spore mass is easily manipulated, so several application techniques work well. Mixing of spores in hydromulch (wood pulp suspended in water) and broadcasting with a tractor-drawn applicator has been particularly effective (31, 42). Container-grown seedlings are also easily inoculated with *Pisolithus* spores (34, 63, 65), with best results when they are dusted onto germinants or young seedlings before competition arises from natural inoculum sources (63). Marx (31) also notes that *Pisolithus* spores have been stored dry for five years at 5°C without apparent loss of viability.

Inoculations with spores of *Rhizopogon* species also appear promising. In Australia, Theodorou (72) and Theodorou and Bowen (74) coated seed of *Pinus radiata* with basidiospores of *R. luteolus*; abundant *Rhizopogon* mycorrhizae formed on seedlings produced from the coated seed. In addition to fresh spores, they also found that freeze-dried and air-dried spores produced mycorrhizae but only at higher inoculum rates. Similarly, Donald (10) used air dried, pulverized sporocarps of *R. luteolus* to successfully inoculate *Pinus radiata* in South Africa nurseries. Castellano and Trappe (unpublished data) found fresh and dried spore suspensions of *R. vinicolor* and *R. colossus* to be effective in inoculating bareroot and containerized Douglas-fir; also, *R. ochraceorubens* spores worked well with containerized *Pinus contorta*, *P. ponderosa*, and *P. radiata*. Their similar experiments with *Gautieria* and

Hysterangium species failed, however, as have attempts by others with various other fungi (31, 80). More needs to be learned about the basic biology of spores before their use in nursery inoculation can be operationally dependable.

The final type of inoculum is pure cultures of ectomycorrhizal fungi. Although many difficulties remain in using this source, techniques for wide-scale application are now being developed (31).

A pure culture of a specific fungus must first be isolated either from the fruiting body or the ectomycorrhiza itself; occasionally, isolates can be obtained from spores, surface sterilized rhizomorphs or sclerotia. The ubiquitous ectomycorrhizal fungus *Cenococcum geophilum* (=*C. graniforme*) is easily isolated from its hard black sclerotia (78). Isolation from sporocarps is, however, easiest for most fungi. Isolations from ectomycorrhizae or rhizomorphs are more difficult, and species often cannot be identified (84). Unfortunately, many ectomycorrhizal fungi grow extremely slowly or not at all in culture. Still, many do grow well in culture, e.g. most species of *Suillus*, *Hebeloma*, *Laccaria*, *Amanita*, *Rhizopogon*, and *Pisolithus*. Consequently, much research attention has been devoted to these species. As improved isolation and culturing procedures are developed, many other fungi can be considered.

Most pure culture inoculation has been restricted to small-scale experiments, although Moser (57, 59) successfully inoculated nursery beds of *Pinus cembra* in Austria with pure cultures of *Suillus plorans* more than 20 years ago. Vozzo and Hacskaylo (83) later used Moser's methods to inoculate pine seedlings in Puerto Rico. Similarly, Theodorou (72) and Theodorou and Bowen (74) inoculated *Pinus radiata* with isolates of *Rhizopogon luteolus*, *Suillus granulatus*, *S. luteolus* and *Cenococcum geophilum*. Marx and Bryan (37) further refined Moser's technique and report excellent results in inoculating nursery beds with *Pisolithus tinctorius* (see Marx (31), for a complete description of these techniques). They grew the fungus three to four months in 2-liter jars containing a sterilized peatmoss-vermiculite substrate moistened with modified Melin-Norkrans nutrient solution (24). After the fungus completely penetrates the substrate, the inoculum is removed from the jars and thoroughly leached with cool, running tap water to remove unassimilated nutrients. The inoculum is spread on the soil at the rate of about 1 l/m^2 and cultivated 8 to 10 cm into the soil. The bed surface is then smoothed and sown. Thorough fumigation of the nursery soil, soon before inoculation preferably with a methyl bromide-chloropicrin mix, is critically needed to reduce competition and antagonism by other soil organisms. Fumigation in autumn before sowing the following spring is often ineffective, because antagonist populations can build up overwinter, especially in areas with cool but mild winters (Trappe and Hung, unpublished data).

Marx (31) recently discussed other pertinent data on development and refinement of pure culture inoculation techniques with *P. tinctorius*. Leached

vermiculite inoculum is heavy from water saturation and is difficult to transport, spread and mix evenly into the soil. After drying leached inoculum to 12 percent moisture at 28–30°C for 56 hours in a forced-air oven, he reports that the dried inoculum performed as well if not better than wet inoculum at several application rates. He attributes this to better mixing of the dried inoculum into the soil. In another nursery study, *Pisolithus* inoculum added at rates of 2.8, 2.16, 1.62, 1.08 and 0.5 $1/m^2$ of soil surface all produced *Pisolithus* mycorrhizae, with 1.08 $1/m^2$ the least amount that was most effective. *Pisolithus* inoculum can also be stored with little loss of viability for seven to nine weeks at 5 and 23°C and for five to seven weeks at 30°C. Marx (31) further notes that *Pisolithus* inoculum can survive in soil for 30 days without a host and maintain inoculum effectiveness. *P. tinctorius* has also been shown to overwinter in nursery plots after seedling removal; seedlings grown the following year in these previously inoculated, then fallow, plots formed abundant *Pisolithus* mycorrhizae. Data of this nature are critically needed to evaluate potential use of other ectomycorrhizal fungi for nursery inoculation.

Inoculation of containerized seedlings with pure cultures also holds great promise (1, 8, 9, 23, 34, 44, 51, 53, 55, 60, 62, 63, 65, 67, 68). A peatmoss-vermiculite mix is used both as an inoculum substrate and container potting mix, so the inoculum is easily incorporated when containers are filled. *Pisolithus tinctorius* has again received the most research attention and has been successfully inoculated on container-grown seedlings in the genera *Pinus* (9, 34, 44, 51, 60, 62, 63), *Pseudotsuga* (44, 51), *Tsuga* (44), and *Quercus* (1, 9, 23, 44, 64). We have effectively inoculated western U.S. species of *Pinus*, *Tsuga*, *Picea*, *Larix*, and *Pseudotsuga* with cultures of *Laccaria laccata* (53, 55, 67, 68), *Cenococcum geophillum* (53, 67, 68), and *Hebeloma crustruliniforme* (68). *L. laccata* and *H. crustuliniforme* appear particularly promising in that the entire root system and substrate are completely colonized by these fast growing fungi. *Thelephora* spp., common natural colonizers of containerized seedlings, can likewise be introduced via pure cultures (62, Trappe and Molina, unpublished data).

High fertility rates, particularly of soluble fertilizers commonly used in rearing containerized seedlings, often restrict or impede mycorrhizal development following inoculation (23, 34, 44, 64, 65, 68); most experience indicates that reduced fertility levels significantly improve mycorrhizal development. Successful mycorrhiza inoculation, however, rarely improves growth of containerized seedlings. Inoculated seedlings often have smaller tops than non-inoculated controls (34, 44, 51, 53, 67). Active fungus utilization of limited host photosynthates impinges on seedling top growth but improves the size and surface of the root system. In addition, some fungi such as *Laccaria laccata* use host photosynthate to form mushrooms in the containers during the first growing season in addition to totally colonizing the root system and substrate (53, 55, 67). Not all fungi respond the same to fertility levels. *Piso-*

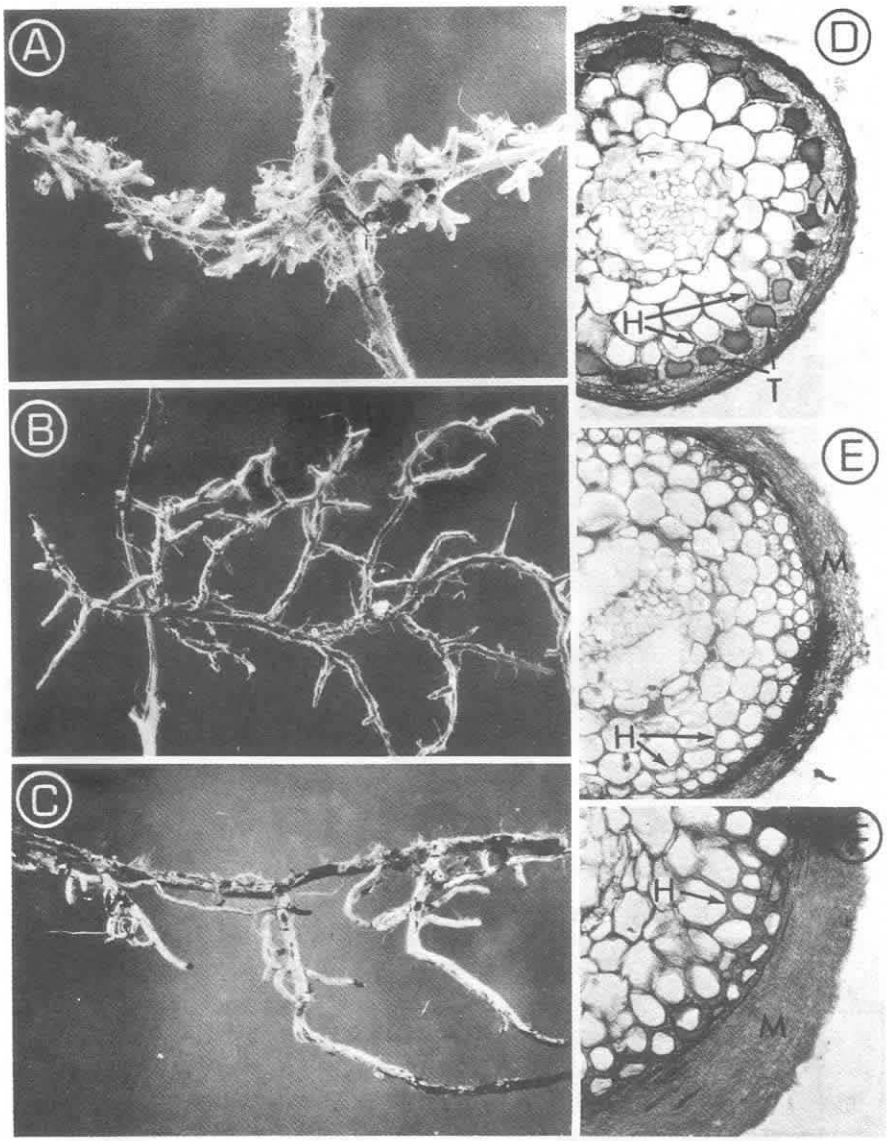

Plate 1. Examples of ectomycorrhizae, form and structure.

A-C: Branching forms and elongation. (A) *Rhizopogon subcaerulescens* + *Pinus contorta* ectomycorrhizae, × 3.3. Note the typical bifurcate (forked) branching typical of *Pinus* ectomycorrhizae. (B) *Rhizopogon ellenae* + *Tsuga heterophylla* ectomycorrhizae, × 3.3. (C) *Paxillus involutus* + *Larix occidentalis* ectomycorrhizae, × 3.3.

D-F: Cross sections of typical ectomycorrhizae (M = Mantle, H = Hartig net, T = Tannin layer). All figures × 175. (D) *Suillus brevipes* + *Pinus ponderosa*. (E) *Astraeus pteridis* + *Tsuga heterophylla*. (F) *Amanita muscaria* + *Larix occidentalis*.

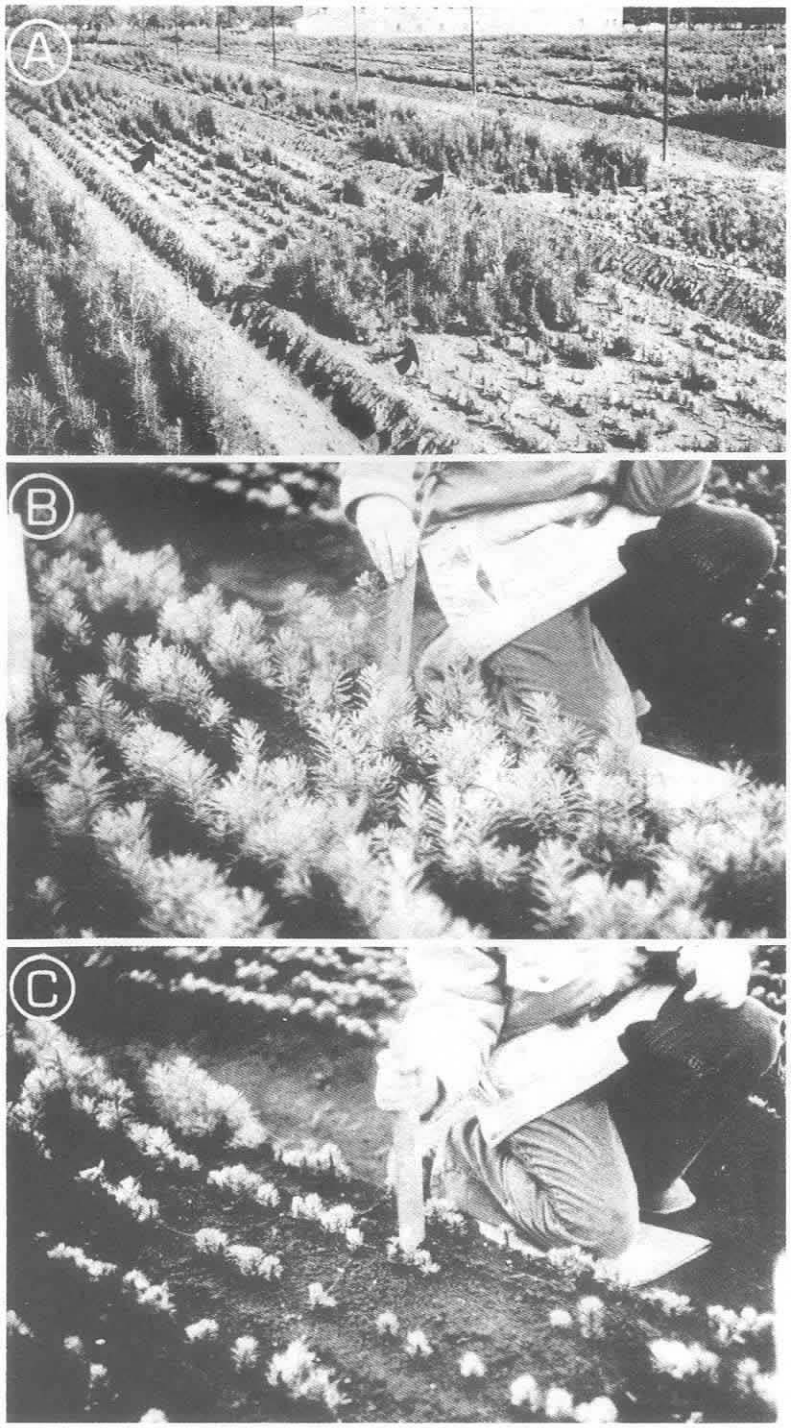

Plate 2. (A) Two-year-old *Pseudotsuga menziesii* seedlings in the Canby Nursery, Willamette Valley of Oregon. Scattered clumps of vigorously growing seedlings (arrows) are mycorrhizal from natural (wind disseminated) inoculum sources [see Trappe and Strand (82)]. (B) First year growth of *Pseudotsuga menziesii* seedlings inoculated with chopped mycorrhizal roots (predominantly *Laccaria laccata* mycorrhizae) at the Humbolt Nursery in northern California. (C) First year seedlings receiving no mycorrhizal inoculation show severe stunting (Figures B and C courtesy of Mike Shrago, U.S. Forest Service).

lithus tinctorius appears especially sensitive to high fertility levels in the rooting substrate (8, 34, 44, 64); high nitrogen levels, however, applied as a foliar mist significantly stimulated *P. tinctorius* development on containerized pine seedlings (8). On the other hand, Molina (unpublished data) found that *Laccaria laccata* inoculum performed equally well regardless of high or low fertility levels. Further research on fertility × mycorrhization interactions is needed to optimize mycorrhiza development and size of containerized nursery stock.

The logistics of producing massive quantities of inoculum presently limits wide-scale use of pure culture inoculum. Large scale production methods are now being developed, however, by Abbott Laboratories*, Long Grove, Illinois. Large volumes of dried *Pisolithus tinctorius* inoculum in a vermiculite carrier are produced quickly in industrial fermentors. A U.S.-wide evaluation of this inoculum has demonstrated it to be effective in producing *Pisolithus* mycorrhizae on several tree species in both bareroot and container nurseries (31, 44). Other firms are also experimentally producing pure culture inoculum of ectomycorrhizal fungi. Industry representatives and mycorrhiza researchers are optimistic that effective commercial inoculum will soon be available on the market.

Selection of fungi for inoculation

The promising outlook for pure culture inoculation raises still another important question: which fungus is best for a particular host or habitat? The need for information on effectiveness of the various mycorrhizal fungi on different host species has been repeatedly emphasized in the literature, yet little data exist. Thousands of ectomycorrhizal fungi and numerous hosts have been reported (77), so careful selection of the best fungi for particular hosts is critical.

Many important criteria must be considered when selecting fungus candidates for nursery inoculation. The major criteria are:

1. Ease of isolation.
2. Growth rate in pure culture.
3. Effectiveness as inoculum.
4. Effects on growth and vigor of host seedlings tops and roots.
5. Ecological adaptability and ecotypic variation.
6. Interactions with soil microorganisms.
7. Host specificity.

Other criteria may be added for special circumstances. It must be stressed

*The use of trade, firm, or corporation names in this paper is for the information and convenience of the reader. Such use does not constitute endorsement by the U.S. Department of Agriculture of any product or service to the exclusion of others which may be suitable.

that many species and ecotypes of fungi are closely adapted to particular habitats, so each isolate must be tested on its own merits. Careful experimentation and good record keeping are essential throughout evaluations of each isolate to document how well it meets the criteria as compared to alternative isolates.

Criteria 1 and 2 are basic to all the others: one must first be able to isolate the particular fungus and grow it reasonably well in culture. Following the experience of Moser (58), we routinely isolate in the field using a small portable hood to reduce air movement. For isolation, we have had best results with small agar slants containing either Melin-Norkrans agar as modified by Marx (24) or potato-dextrose agar.

Relatively fast growing fungi are generally preferred for inoculation because of their short incubation period. Unfortunately, many otherwise desirable ectomycorrhizal fungi grow slowly. As the physiological growth requirements of mycorrhizal fungi become better understood, growth of the slow-growers may be improved for use in inoculation. Fungi that do not grow or grow only slowly in culture may be highly specialized in their symbiotic relationship to the host and may benefit their host greatly. Clearly, further study of these recalcitrant fungi is needed.

Marx (31) emphasizes two additional points about culture characteristics. First, fresh cultures are preferred to cultures repeatedly transferred and stored for several years. Some ectomycorrhizal fungi lose their mycorrhiza-forming capacity after longterm storage, presumably due to adaptive enzyme shifts in utilizing artificial substrates. This can be overcome for many fungi by storing them under refrigeration in sterile water for several years without transferring (41). Marx (31, 32) further suggests passing important fungus cultures through a host, i.e. host inoculation and mycorrhiza formation followed by reisolation, every few years to maintain mycorrhiza-forming capacity. *Pisolithus tinctorius* isolates used for wide-scale inoculations have significantly improved in mycorrhiza-forming capacity and enhancement of seedling growth following repeated reisolations (32). Secondly, fungi which produce large hyphal strands or rhizomorphs in culture or soil may be superior in soil exploration and mineral uptake to those which lack rhizomorphic growth (3, 4, 31). For example, Bowen (3, 4) suggests that the extensive rhizomorph network of *Rhizopogon luteolus* is largely responsible for enhanced nutrient uptake and seedling growth. Marx (31) believes that hyphal strands of *P. tinctorius* not only enhance nutrient uptake but also increase seedling survival potential under adverse conditions. Schramm (66) and Marx (28) have traced mycelial strands of *P. tinctorius* through coal spoils up to 4 m from seedlings to sporocarps. Comparable data and observations are needed for the many fungi which do not form such hyphal structures.

Criteria 3 and 4 are next considered in the selection process. After the fungal inoculum has been prepared, its effectiveness must be determined.

Feeder roots susceptible to mycorrhizal colonization do not form on seedlings until six to eight weeks after seed germination. During this period the vermiculite particles are believed to provide a protective niche for the naked mycelium. Survival and effectiveness of the inoculum is determined by examining roots for ectomycorrhizal formation. Roots should be sampled periodically during the first growing season. The numbers and kinds of other native mycorrhizal types should also be noted to assess effectiveness of the soil fumigation and the competitive ability of the inoculated fungus. Marx (31) emphasizes that the candidate fungi must be aggressive in forming mycorrhizae as soon as feeder roots are produced and in maintaining superiority over native fungi in the nursery. With *Pisolithus tinctorius*, maximum benefit for pine seedlings results only after at least two-thirds of the feeder roots are colonized by *P. tinctorius*. All nursery cultural practices should also be carefully monitored and recorded for future reference.

The most crucial criterion in the selection process deals with the benefits the host derives from inoculation with a specific symbiont. Differences between inoculated and noninoculated seedlings in height, top and root weights, and stem caliper must be compared. Marked improvement in bareroot nursery seedlings can be expected by effective inoculation with a highly beneficial fungus. Because the seedlings are raised for forestation purposes, however, the critical test is survival and growth after outplanting as compared to normally produced nursery stock. Survival and growth data must be collected over at least the first three years. Nurserymen will not want to inoculate seedlings unless it significantly improves nursery production or field performance. Readers are referred to Marx (31) for a detailed review on current performance of outplanted inoculated nursery stock.

Criterion 5 deals with fungal physiology with special reference to ecological adaptability. Field observations as well as laboratory tests are important. Data should be recorded on the ecological range of the fungus as well as specific habitat types in which it is found. Environmental conditions of outplanting sites also need consideration. Planting sites characterized by drought or temperature extremes are commonly difficult to reforest. Trappe (unpublished data) has found that conifer seedlings inoculated with *Pisolithus tinctorius* in Oregon survive better than "nursery run" seedlings on hot, droughty sites. *Pisolithus* did not improve performance of seedlings on cool, high elevation sites, however. In mine spoils soil toxicity is a major problem. Temperature and moisture requirements of the fungus can easily be estimated from simple laboratory tests (80). Our working hypothesis is that fungi already adapted to conditions similar to the planting sites should be the primary candidates for inoculation. Trappe (80) states: "In essence, the provenance of the fungus should be considered along with the provenance of tree seed."

Special emphasis should be placed on the ecotypic variation displayed within fungal species (80). For example, Molina (51) found that six isolates

of *Pisolithus tinctorius* differed significantly in ability to colonize feeder roots of container-grown Douglas-fir and lodgepole pine seedlings. Marx (32) provided further evidence for worldwide variation among *P. tinctorius* isolates and made several suggestions for selecting the best strain. On the other hand, Molina (55) recently noted that several isolates of *Laccaria laccata* lacked significant differences on mycorrhiza colonization following inoculation of four conifer species; all isolates completely colonized the root systems and sporocarp production was prolific. Thus, each fungus isolate must be tested on its own merit and with several performance criteria in mind.

Criterion 6 has received scant attention in selection of fungi for nursery inoculation, but it has potential. Ectomycorrhizal fungi protect host roots to varying degrees against certain pathogens (25). Nurseries with continuing root pathogen problems may wish to introduce mycorrhizal fungi selected for the ability to protect seedling roots from disease. For example, *Laccaria laccata* protected Douglas-fir seedlings against *Fusarium oxysporum* even in the absence of mycorrhiza formation (69, 71). Recent work by Bowen and Theodorou (5) on interactions of ectomycorrhizal fungi and bacteria emphasizes the need to assess compatibility and potential antagonisms of the resident microflora with the introduced fungi.

The final criterion is host specificity. Many ectomycorrhizal fungi exhibit wide host ranges: *Amanita muscaria*, *Boletus edulis*, *Laccaria laccata*, *Pisolithus tinctorius* and *Cenococcum geophilum*, to mention a few. Others are more restricted. Some are known only to fruit in association with a single host or genus of hosts. The association of *Suillus grevillei* with *Larix* species and *Leccinum scabrum* with *Betula* species are two commonly cited examples. The genus *Pinus* appears to have its select group of "pine" mycorrhizal fungi. *Pseudotsuga menziesii* has many mycorrhizal fungi common only to its distribution. Precise data of this nature is very important if we plan to inoculate a wide range of tree species, especially in regions where many different host genera are raised commercially. The Pacific Northwest of the U.S.A., for example, contains at least 16 native genera of ectomycorrhizal hosts, including over 60 species. At least a third of these are raised in bareroot and container nurseries. A single nursery may raise 10 species. Should many different fungi be inoculated to satisfy the different hosts, or is it better to inoculate with one fungus capable of colonizing them all? Mikola (49) believes that, due to its more specialized relationship with a particular host, a host-specific fungus would benefit its host more than would a broad-ranging fungus. This hypothesis warrants further research, especially with the development of wide scale, pure culture inoculation.

Pure culture synthesis of mycorrhizae as developed by Melin (46) and modified by Hacskaylo (13) and others provides good evidence on host specificity. Seedlings are raised aseptically in two-membered culture with an introduced mycorrhizal fungus. With large glass test tubes for the chambers,

numerous combinations of host species and fungi can be readily assessed in three to six months when the seedlings are harvested (52, 54). With this technique we have found that fungus-host specificity is more complex than simply a constant association of sporocarps with particular hosts in the field (52, 54, 56). Some fungi that fruit with specific hosts form typical ectomycorrhizae with other, unassociated hosts.

The *Pisolithus* story: A case in point

The intensive research on *Pisolithus tinctorius* conducted by Dr. Donald Marx and co-workers at the U.S. Forest Service Institute for Mycorrhizal Research and Development, Athens, Georgia, has gathered the support of both the research community and industry and has brought mycorrhizal applications in forestry to the forefront. Although focusing on one fungal symbiont, *P. tinctorius*, their underlying hypothesis is that growth and survival of seedlings can be significantly improved by inoculations with specific mycorrhizal fungi. A brief summary of their work follows with special emphasis on their integrated use of many of the concepts presented in this paper (see Marx (31), for a more detailed review).

Schramm (66) reported the extensive development of *P. tinctorius* ectomycorrhizae and sporocarps associated with pine roots growing on anthracite mining wastes in Pennsylvania. *P. tinctorius* was often the pioneering mycorrhizal symbiont on the young, most vigorous pine seedlings. Realizing that the extremely high soil temperatures reported by Schramm might limit fungal symbionts to a few adapted species, Marx *et al.* (40) explored the temperature-growth interactions of *P. tinctorius*. They found that it formed more ectomycorrhizae with *Pinus taeda* seedlings at 34°C than at lower temperatures; mycelial cultures grew at temperatures as high as 40°C. Marx and Bryan (35) later found that aseptically grown *Pinus taeda* seedlings colonized with *P. tinctorius* survived and grew as well at 40° as at 24°C; comparative nonmycorrhizal seedlings and those colonized with the fungal symbiont *Thelephora terrestris* had less survival and did not grow at 40°C. Clearly, the adaption to higher temperatures was a major factor in allowing *P. tinctorius* to invade the coal wastes.

Realizing the practical significance of these results, Marx (26) and co-workers surveyed strip-mined lands for the presence of *P. tinctorius*. They found it to be the dominant and often only ectomycorrhizal fungus of pine roots growing on coal wastes in Indiana, Pennsylvania, Ohio, West Virginia, Virginia, Kentucky, Tennessee, and Alabama and on kaolin soils in Georgia.

These results prompted extensive investigation of ways to inoculate and establish *P. tinctorius* ectomycorrhizae on roots of pine seedlings destined for outplanting on mine spoils. Marx and Bryan (37) developed techniques as previously described for preparing pure culture inoculum and inoculating

nursery soil with *P. tinctorius*. They report excellent success in establishing *P. tinctorius* in the nursery with doubled growth of inoculated seedlings over uninoculated controls (38). Inoculation with *P. tinctorius* basidiospores has also succeeded (27, 38, 42). More importantly, *P. tinctorius* inoculation has significantly increased survival and growth of seedlings on mine spoils (2, 26, 30, 33, 62). Many of these sites had a history of repeated failures of pine plantations. *P. tinctorius* ectomycorrhizal colonization can also increase survival and growth of southern pines on routine reforestation sites (39).

Development of the *P. tinctorius* inoculation program included many of the selective criteria discussed previously. *P. tinctorius* is easily isolated from sporocarps and grows rapidly in culture. Inoculation with pure cultures of vegetative mycelium as well as basidiospores often results in complete colonization of the entire feeder root system. Overall host response and performance in both nursery and plantation are excellent. Both field and laboratory studies emphasize the ecological adaptiveness of *P. tinctorius* to stressful sites, including tolerance to high soil temperatures, moisture stress, and soil toxicity. Feasibility of utilizing commercially produced *P. tinctorius* inoculum is being studied and appears promising. Finally, *P. tinctorius* is distributed worldwide and forms ectomycorrhizae with over 48 species of trees (29). Worldwide use of this highly beneficial fungus is quite possible and experimentation in many regions is underway (50).

These impressive results and the concepts they represent have stimulated inoculation research programs around the world. Clearly, with the increased demand for and dwindling supply of wood resources, the regeneration of cut-over lands and establishment of man-made forests is of highest priority. Inoculation with highly beneficial mycorrhizal fungi specifically selected for certain traits can enormously increase the chances of meeting this priority.

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